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On the Changes in the Proteids in the Seed Which Accompany Germination

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III. *On the Changes in the Proteids in the Seed which accompany Germination.*

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A curious and characteristic feature of the life-history of the higher forms of plants is the long resting period which takes place in the seed, following the reproductive act, after a certain amount of development. Fertilisation of the female element is succeeded by a period of activity, during which great changes in the oosphere take place, many new cells are formed, and the new individual becomes recognisable. But then comes a remarkable alteration; the development for a time is arrested, no new cells are produced, but those already formed, which constitute chiefly the first leaves or cotyledons of the new plant, become filled with nutrient materials, forming reserves upon which, after the resting period, the young plant will subsist, and which will enable it to resume its growth. Or it may be that the nutrient material may be accumulated in cells immediately surrounding the young embryo, cells which form the so-called endosperm and which are not actually part of it. A curious feature this, not represented exactly by anything in the cycle of animal life, though perhaps the condition of the egg which is deposited by the parent and quickened later into active life approaches somewhat to it. This differs greatly, however, in the length of the quiescent period, which in the seed may be almost indefinitely prolonged. What changes, if any, take place in the cells during this period is not known and cannot well be ascertained. At the end of this time changes do take place, and the arrested development is resumed. That the condition of things inside the seed is not exactly alike always, seems pointed to by the fact that seeds of the same plant do not germinate at all times with equal readiness, though exposed to the same favourable conditions. During this period, long or short as it may be, and its length varies extremely, the different bodies occupying the interior of the cells of the cotyledons or the endosperm maintain their character apparently unchanged, or, if changed at all, the nature of the change is such as not to be recognisable by microscopic examination or by chemical analysis, only being marked by greater or less resistance to the setting up of the manifest changes which are known as the process of germination.

The process of deposition of the several reserve products in the cells of the seed has been watched by many observers, and the details of the storage have been examined

and traced out step by step. The resumption of the arrested development, under the conditions of moisture and of temperature which we call favourable to germination, involves intricate metabolic processes in which the different materials that have been stored are all separately concerned, each group of bodies being transformed into nearly related ones which are adapted for the new conditions of life. Instead of the resting forms of proteids, carbohydrates, &c., which are not diffusible and hence cannot pass from cell to cell and so traverse the plant, we have new forms which can readily travel to those points where growth is proceeding and new cells are being formed, and hence plastic material is required. The details of these transformations are in many cases still obscure, though some facts have been ascertained which throw light upon the nature of some of the chemical processes. In the case of the starch, which is so constant a constituent in seeds, it has been proved that the formation of sugar takes place from it by the agency of a ferment, exactly as it does in the corresponding process in the animal economy. From almost any seed a so-called diastatic ferment can be obtained, and so constant is the occurrence of the latter body in vegetable organisms that it can be prepared from almost any part of plants. From analogy it would seem probable that the proteolytic changes noticeable would have a similar cause, and that from seeds in which large quantities of reserve proteids are stored evidence of such a body could be obtained.

Other seeds are noticeable in connection with the large store of cellulose which they contain in their endosperm cell-walls, a store which disappears as the process of germination proceeds, and which is no doubt made use of for the nourishment of the young plant. It seems *possible* at least that a similar ferment action may be the cause of the transformation here, particularly when it is remembered that in the intestines of many animals, notably in the herbivora, a digestion of cellulose somewhere takes place, and that probably under the influence and by the activity of bacteria, which are themselves vegetable organisms, although in their case it is hardly likely that an *isolable* ferment is prepared by them for the work. The proved existence, therefore, of diastatic ferments almost universally in plants, and the probability of existence of the others alluded to, besides the discovery of other ferments in the vegetable kingdom, not immediately connected with the process of germination, have directed investigation into the metabolic changes in the seed with the view of discovering such ferments there. In 1874 some observations were published by v. Gorup-Besanez, which indicated the existence of a peptone-forming ferment in the seeds of the Vetch, being there in company with another which had amylolytic or diastatic properties. V. Gorup-Besanez speaks of this body* as having power to convert fibrin into peptone, but he did not apparently trace its normal action in germination, as he does not indicate what changes it causes in the proteids stored in the seed. In 1875 he supplemented his observations on the Vetch in another paper,† in which he states

* 'Deutsch. Chem. Gesell. Ber.,' 1874, p. 1478.

† *Ibid.*, 1875.

he has discovered a similar body in the seeds of Hemp, Flax, and Barley. V. Gorup-Besanez was followed in 1878 by Krauch,* who says that he fails to confirm his results. In a later paper by the latter writer,† he severely criticises v. Gorup's method of working, and states that the results he obtained were due to imperfect experiments. While v. Gorup-Besanez says that fibrin acted on by the body he prepared from the vetch seed was dissolved, and that then the solution when filtered gave a good biuret reaction, Krauch insists that the biuret reaction was due to something present in the ferment solution and was readily yielded by the latter alone. He says, further, that the fibrin in his own experiments appeared to get less, but that this was due to shrinkage of the flocks of it, and not to solution. Krauch's work, however, appears untrustworthy, for he does not explain the disappearance of the fibrin which v. Gorup alleges, nor does he show that there was no formation of peptone in the process, although the ferment solution itself might have given a biuret reaction. With such a ferment solution to begin with, other means were necessary to detect the peptone if any were formed. Krauch's own control experiments were somewhat scanty.

Proteolytic Ferments.

The nature of the ferment discovered by v. Gorup-Besanez was not satisfactorily established in his investigations. He calls attention himself to the fact that in the young shoots of newly-germinated plants of Vicia, under certain conditions, large quantities of leucin and asparagin might be shown to exist. The ease with which crystalline bodies of that nature would be able to pass through such structures as cell walls points to the probability of this rather than peptone being the form in which nitrogenous matter would pass to the growing points from the reservoirs in which it had been stored. Moreover, the fact that peptone cannot be discovered in or near the growing parts, and the almost complete indiffusibility of any other form of proteid matter, lend much support to the view that crystalline products are the ultimate expression of germinative metabolism of proteids. On these grounds, therefore, there is a great probability that the proteolytic ferment in the seeds will be found to resemble the tryptic rather than the peptic ferment of the animal organism. V. Gorup-Besanez was not, however, able to convince himself that the ferment in the vetch seed carried the changes in the fibrin beyond the stage of peptone.

Since that time Martin‡ has shown that a tryptic ferment is present in the latex of Papaya carica.

When v. Gorup-Besanez was writing, but little had been ascertained as to the nature of the proteid substances stored in seeds. Ritthausen's work had apparently shown the existence of bodies differing greatly from animal proteids, and forming a

* 'Beiträge zur Kenntniss der ungeformten Fermente in den Pflanzen.' Berlin, 1878.

† 'Landwirthsch. Versuchs-Stat.,' vol. 27, p. 383.

‡ 'Journ. of Physiol.,' vol. 5 (No. 4), and vol. 6 (No. 6).

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series peculiar to vegetable organisms. Inasmuch as these were not shown to approach the animal proteids in any particular direction, and on account of the paucity of the reactions known to characterise them, nothing was done in the direction of ascertaining their fate during germination.

In 1877* and 1880† Weyl and Zöller began to place them on the same footing as animal proteids, and to show how they resembled them, establishing the existence of globulins in seeds. About the same time a series of elaborate investigations, by Vines,‡ cleared up many points of difficulty, and gave us for the first time a clear conception of the chemical nature of these reserve vegetable proteids which exist in the seed in the form of the so-called aleurone grains. According to the latter observer, these consist of members of the great groups of the albumoses and the globulins.

The nature of the aleurone grains, or stores of vegetable proteids, now being, at any rate generally, understood, it becomes possible to ascertain something about the nitrogenous metabolism of the process of germination as a whole; to see whether it is a process of ferment action, for this really can hardly be considered established, though rendered highly probable, by v. Gorup-Besanez's experiments on fibrin, the aleurone being greatly different from this form of proteid; to ascertain whether, if so, the action can go as far as the formation of crystalline nitrogenous bodies; and to trace the series of changes in the proteids which take place as the germination proceeds.

During the past year I have endeavoured to deal experimentally with these questions, and for the purposes of the investigation have taken the Lupin as, for many reasons, the most suitable. The seeds are of large size, and germinate very readily; from Vines's work the approximate composition of the proteid reserve materials is very well known; and according to v. Gorup-Besanez, in plants nearly related, *i.e.* the Vetches, a proteolytic ferment exists. In his paper already alluded to, the latter writer states that with the Lupin he only obtained a negative result; but, for the reasons mentioned, it seemed not impossible that such a ferment existed.

The products of the decomposition of fibrin by proteolytic ferments being well understood and easily recognisable, my first experiments were directed to the action of the extract of the seeds on this body. A considerable quantity, about a quarter of a peck, of the seeds of *Lupinus hirsutus* were germinated for four days in a green-house, and when the radicle had grown to a length of about 2–3 inches they were removed, the cotyledons separated from the other parts, and ground in a mill. V. Gorup-Besanez prepared his extracts by an elaborate process of dehydration by alcohol, extracting with glycerine, &c., several times repeated.§ This method was

* 'Zeitschr. f. Physiol. Chem.,' vol. 1, 1877. 'Deutsch. Chem. Gesell. Ber.,' Jahrg. 13, 1880, p. 367.

† 'Deutsch. Chem. Gesell. Ber.,' Jahrg. 13, 1880, p. 1064.

‡ 'Journ. of Physiol.,' vol. 3 (No. 2).

§ His extract so prepared would yet contain a large amount of albumose which is soluble in glycerine

found to answer well with Vetch seeds, but did not, for some reason, extract anything from the Lupin. Instead of following it, I only made a glycerine extract of the ground germinating seeds, which I used after dialysing for some time. In consequence of this mode of extraction it was necessary to modify the ordinary method of testing its activity. This glycerine extract would not only contain any ferment present, but would have taken up such of the proteids of the seed as were soluble in water. According to VINES, in the Lupin there occur hemialbumose and a form of globulin. The latter is soluble only in salt solutions, but the former dissolves in water readily. As this hemialbumose also gives the biuret reaction, which is always relied on as the characteristic reaction of peptone, it is evident that its presence in the extract containing the ferment would render it very difficult to say that the latter formed peptone at the expense of the fibrin, unless some method could be devised which should separate the albumose from any peptone that might be formed, or a reaction discovered which peptone gives and albumose does not. Such a method of separation, neglected apparently by both v. GORUP-BESANEZ and by KRAUCH, is furnished by dialysis. According to VINES, hemialbumose does not dialyse, while peptone does so readily. It is evident, therefore, that if a mixture of the two are at any time present in the same dialyser the peptone will pass through and be found in the dialysate, while the hemialbumose will not. Before trusting to this method, I carefully tested this asserted indiffusibility, and found I could confirm VINES completely. After more than a week's exposure of a solution of hemialbumose in a parchment-paper dialyser, the liquid outside failed to give a biuret reaction, while the solution inside did so readily. Instead of using glass vessels, therefore, for my digestive experiments, I carried them all out in well tested dialysing tubes, through the walls of which would pass, as formed, the peptones and nitrogenous crystalline bodies, should such be produced, while any hemialbumose or globulin present in the extract would be retained in the vessel. In all cases careful control experiments were carried out, all the conditions being the same in both sets, except that one set contained the ferment extract and the other did not. The dialysers, too, were carefully tested as to their intactness at frequent intervals. The fluid outside was made of the same reaction as that inside.

The experiments on which I base my conclusions were carried out in nearly every case with extracts which had themselves been dialysed with care. The reason for this was that, as germination had begun in the seeds, it was probable that in the extracts there would be a small amount of leucin or asparagin. The dialysis was continued till the dialysate gave no proteid reaction, and on concentration and evaporation on a glass slide deposited no crystals. In one or two cases extracts were used without such dialysis, but when this was the case precautions were taken against error, which will be detailed in giving the results.

In selecting the medium in which to conduct the experiment on fibrin, attention

and is not coagulated by exposure to alcohol, remaining under it unchanged for a considerable period. This gives the biuret reaction, and explains, therefore, KRAUCH's criticism.

was first given to the reaction of the germinating seed. This was found to be faintly acid, and consequently a fluid containing free HCl to the amount of ·2 per cent. was used. Further experiments bearing on this point will be detailed later. It was, unfortunately, not possible to reproduce the conditions obtaining in the seed, where the proteid matter is in considerable excess and but little fluid is present.

Some fibrin was taken and boiled for twenty minutes in weak HCl, to destroy any possible ferment adherent to it. It became swollen up and of the usual semi-transparent appearance. A quantity was put into a dialyser with about 30 c.c. of the dialysed glycerine extract, which was mixed with an equal bulk of ·4 per cent. HCl; and the dialyser was immersed in a beaker containing 100 c.c. of ·2 per cent. HCl. A control was kept by having a precisely similar quantity of the glycerine extract boiled before adding the fibrin, and another by putting a similar quantity of fibrin into ·2 per cent. HCl alone. All were then placed on a water bath at 37–40° C., and left to digest. After a period of time, varying in different experiments, but in every case very much more prolonged than is necessary with gastric or pancreatic extracts, the dialysate from the tube which contained the unboiled extract of the seeds was found to contain peptone, as evinced by the pink colour given on addition to it of excess of NaHO and a drop of $CuSO_4$. When this had been well marked for some time, the dialysate was boiled. No turbidity resulted, nor did any opalescence or precipitate appear on neutralisation. Alcohol gave a precipitate which settled out slowly. On concentrating the dialysate, and evaporating slowly, crystals were formed which were recognisable under the microscope as those of leucin. They were carefully compared with the crystals of leucin figured by Funke in his 'Physiological Atlas,' and corresponded entirely with them.* When the liquid containing these was evaporated to dryness with HNO_3, and the residue treated with caustic soda, and again evaporated, it gave the oily drop said to be characteristic of leucin (Scherer's test).

Besides these, others were present in less quantity, which, from their form, appeared to be those of tyrosin. The liquid containing them changed to a pale pink colour when boiled with Millon's reagent. This was confirmatory of the presence of the latter body, though it cannot be held to be by itself conclusive, as a trace of peptone present, if not enough to be precipitated by the Millon's reagent, would give a somewhat similar reaction.

No digestion took place in either of the control tubes.

The process was continued in this case for six days. In another experiment I worked with an extract that had not been dialysed, when I proceeded rather differently. In the dialyser I put 30 c.c. of the extract and 30 c.c. of ·4 per cent. HCl. In the beaker in which the dialyser was immersed I put 120 c.c. of ·2 per cent. HCl. After 24 hours I poured this away and substituted 120 c.c. of fresh HCl ·2 per cent. I changed this again after 24 hours more, and again after a further

* The microscopic slides of these crystals were further examined for me by Dr. Sheridan Lea, who kindly allows me to quote his opinion that they were those of leucin.

24 hours. This treatment removed any crystalline bodies present in the extract used, leaving in the final dialysate only such as had been formed in the latter part of the digestion. The quantity found in the last 120 c.c. was as great as in the former experiment. On watching from time to time the progress of the action, as shown by the changes in the fibrin, a correspondence was evident between the behaviour of the lupin extract and that of pancreatic juice. True, the reaction of the fluid was different, but the fibrin seemed to be eaten away from the outside just as in pancreatic digestion, and not to gradually pass into solution, as it does when acted on by pepsin. When the digestion was complete the liquid was quite turbid, and deposited, on standing, a sediment of fine particles. In the tubes in which the fibrin was only treated with HCl it maintained throughout the peculiar, almost translucent, appearance characteristic of it almost immediately it is subjected to the acid's action, and the liquid was only slightly opalescent after days of treatment.

In no case during the experiments did bacteria appear in any of the tubes.

The course of digestion as estimated by the products formed also closely resembled that brought about by trypsin. In the process caused by the latter there are three distinctive bodies or groups of bodies formed apparently successively. The first of these is precipitated on neutralising the digesting mixture, and the precipitate is soluble in either weak acids or alkalis. To it has been given the name of parapeptone. Very soon after digestion has begun, the so-called peptone is recognisable, and leucin and tyrosin, crystalline bodies, appear. Besides these, there are formed, simultaneously apparently with the parapeptone, bodies called albumoses, which possess the peculiar properties of being insoluble, some in slightly acid solutions, some in water, at ordinary temperatures, but being soluble at temperatures higher than 70° C. In the digestions by the lupin extract the liquid in the dialyser very soon gave a conspicuous neutralisation precipitate, soluble in acids or alkalis, and being evidently parapeptone. Besides this there appeared to be a considerable amount of albumoses formed, more than is usually seen with either pepsin or trypsin. These were precipitated with the parapeptone, but could be readily separated from the latter by warming the tube containing the mixed precipitates suspended in water. A good deal of the suspended matter dissolved, and, on filtering while hot, the insoluble parapeptone was removed, while the albumose remained in solution and was thrown down again as the liquid cooled. The albumose in greatest quantity here differed somewhat from the hemi-albumose of Kühne, or α-peptone of Meissner, as this body is precipitated by acids. It corresponds more closely with Kühne's heteroalbumose,* which is insoluble in water, being thrown down by dialysis. A certain amount of dysalbumose also was present.

According to Kühne, the albumoses are partly the result of the action of the acids on proteids, for they are formed by digesting fibrin with HCl ·2 per cent. for a considerable time. In this case they were not so formed, but were due to the action of the extract, for a control tube with HCl only and fibrin contained a mere trace of them.

* Kühne and Chittenden, 'Ueber Albumosen,' 'Zeitschr. Biol.,' vol. 20, 1884, p. 11.

The subsequent or coincident appearance of peptone and crystalline bodies has already been described. For the satisfactory demonstration of the formation of these, an experiment was made on a large quantity of fibrin which was subjected for a week to the action of 25 c.c. of the extract. At the conclusion of this time the method used to separate leucin by v. Gorup-Besanez in his investigations was followed. The liquid was boiled, filtered, and neutralised; the neutralisation precipitate removed, and acetate of lead added. This body forms a compound with leucin which is insoluble in alkaline fluids. The precipitate so formed was filtered off, and suspended in water. The leucin and lead compound was then decomposed by passing a stream of SH_2 through it, and the sulphide of lead so formed removed by filtration. The filtrate was evaporated to dryness and extracted with boiling absolute alcohol. The last reagent would take up leucin, but not any traces of proteids that had gone down with it. The alcohol extract was then evaporated to concentration, when it deposited the crystals. The essential nature of the action of the extract on fibrin having thus been established, it becomes possible to speak of the presence in it of a proteolytic ferment and to consider this a tryptic rather than a peptic one.

Several points connected with its action were then investigated.

1. *At what temperature is it most active?*

The influence of temperature on the ferments of the animal organism is one of the most remarkable features they possess. Their action is suspended at a very low degree, gradually improves up to an optimum, which is the temperature of the animal body, and beyond this point declines, till on exposure to about 70° C. they are destroyed.

It does not seem improbable that, as the proteolytic ferment of the Lupin works naturally in a body which is not at so high a temperature as that occurring in the alimentary canal, a lower degree of heat than that would suit it best. In the experiments on the point 20 c.c. of the dialysed glycerine extract were taken and diluted with 20 c.c. of HCl of ·4 per cent. strength. This was then divided into two, and a measured quantity of boiled swollen fibrin was placed in each. One was kept at the temperature of the laboratory, and the other put in a water-bath at a temperature of 37° C. After two days' digestion three-quarters of the fibrin in the latter had been digested; the liquid was turbid, and peptone in abundance present. In the former, digestion was just beginning to be evident, the edges of the fibrin only being corroded away. Two days later the warm tube contained no recognisable fibrin, while the cool one showed digestion about half completed.

Boiling the fluid which contained the ferment effectually destroyed its activity.

It therefore corresponds in its behaviour to the animal ferments, working best at a moderately high temperature, such as 40° C., but being destroyed by too great heat.

2. *What is the medium most suitable for its action?*

In the germinating seed, as already stated, the reaction was acid, the depth of tint given to very sensitive litmus paper being about the same as that caused by ·2 per cent. HCl. As the tryptic ferment in the pancreas requires an alkaline medium for its activity, experiments were made to test whether this one worked best in an alkaline fluid. Six tubes were taken, of the same capacity, and into each was put a measured quantity of boiled swollen fibrin. The tubes were labelled A, B, C, D, E, F, and to the fibrin in each was added as under:—

A. 50 c.c. ·2 per cent. HCl and 5 c.c. ferment extract.
B. 50 c.c. ·4 per cent. HCl and 5 c.c. ferment extract.
C. 50 c.c. 1 per cent. HCl and 5 c.c. ferment extract.
D. 50 c.c. 1 per cent. Na_2CO_3 and 5 c.c. ferment extract.
E. 50 c.c. 1·5 per cent. Na_2CO_3 and 5 c.c. ferment extract.
F. 50 c.c. ·5 per cent. Na_2CO_3 and 5 c.c. ferment extract.

Three control tubes were also prepared, containing no ferment extract.—

G. 50 c.c. ·2 per cent. HCl.
H. 50 c.c. ·4 per cent. HCl.
I. 50 c.c. 1 per cent. Na_2CO_3.

To each of the latter an equal amount of fibrin was put, as in the first six. After 24 hours, and again after 48 hours, they were examined, with the results given in the subjoined Table.

	After 24 hours.	After 48 hours.
A (·2 per cent. HCl) . .	Digestion most active. Most peptone formed .	Fibrin all gone
B (·4 per cent. HCl) . .	Digestion begun. Less active than A	Fibrin about half gone
C (1 per cent. HCl) . .	Digestion begun. Nearly as fast as B	About as B
D (1 per cent. Na_2CO_3) .	No change in fibrin, except that it had lost translucency. No peptone formed. A little alkali-albumin present, due to the Na_2CO_3	No further change evident
E (1·5 per cent. Na_2CO_3) .	As D	As D
F (·5 per cent. Na_2CO_3) .	As D	As D
G	No change, but formation of a little acid-albumin by the acid	No further change
H	As G	No further change
I	No change, except that fibrin was shrunken and a little alkali-albumin formed	No further change

Another set of tubes, prepared similarly, confirmed these results. The medium in which the ferment acts most advantageously is therefore a weak acid, about equal to ·2 per cent. HCl.

3. *What is the influence of alkalis and neutral salts on the ferment?*

The complete absence of any sign of activity in the alkaline tubes suggested, as the third point, an enquiry as to what had happened to the ferment in these; that is, had its activity been merely suspended, or had it been destroyed as is the case on similar treatment of pepsin?* To determine this point, which had rather an important bearing on subsequent work, the two tubes D and F in the last series of experiments were made the subject of further investigation.

D contained the ferment in 1 per cent. Na_2CO_3 solution. It had been shown to be inoperative on fibrin in such a fluid. If its action were only suspended, it would be able to digest fibrin if the reaction were made acid to about the extent of ·2 per cent. HCl. If it had been destroyed, such treatment would not restore the activity. At the same time, by the neutralisation of the Na_2CO_3, some salt would be formed which might or might not have an influence on its rate of activity, should such exist.

The contents of the tube were therefore carefully neutralised, and the alkali-albumin referred to consequently precipitated. It seemed possible that any ferment present might be carried down by this precipitate, as such bodies do generally accompany precipitates in the fluid in which they are present. Only half the liquid was consequently filtered. Both were then added to an equal bulk of ·4 per cent. HCl, and a fresh measure of fibrin added to each. The little quantity of the dissolved alkali-albumin in the unfiltered tube would not interfere with the result, as the activity was judged of by the disappearance or not of the fibrin. The filtered tube was labelled D_1 and the unfiltered one D_2.

It was of course necessary to control the experiment by having two precisely similar tubes containing ferment that differed from these only in not having been exposed to the action of the alkali. It was necessary, therefore, to add to these exactly the same amount of neutral salt which D_1 and D_2 contained. This was done as follows:—

50 c.c. of Na_2CO_3 solution 1 per cent. were taken, and 5 c.c. dialysed ferment extract added. This reproduced the condition in D at commencement of the former experiment. Instead of allowing the alkali to act on the ferment, the mixture was at once made neutral. There was present now the same amount of salt as in D after digestion and subsequent neutralisation. The liquid was a little turbid from presence of a little proteid matter in the extract used. Half of it was filtered and half left unfiltered. 27·5 c.c. of ·4 HCl and a measure of fibrin as before were now added to each; they were labelled D_3 and D_4, and the whole set placed on a water bath.

The digestion was tested as it proceeded, but no marked results were obtainable for a longer time than usual. The digestion was allowed to proceed for 54 hours, when the following results were noted:—

*. Langley, 'Journ. of Physiol.,' vol. 3, p. 246.

	D_1	D_2	D_3	D_4
After 24 hours	No apparent change	No apparent change	Digestion beginning. Turbidity setting in.	Digestion beginning. About as D_3.
After 54 hours	No apparent change. Quantity of fibrin not lessened at all. Liquid quite clear.	No apparent change. (Unfortunately broke this tube in removing it, so did not measure the fibrin. To the eye there was no change.)	Liquid very turbid. About a quarter of the fibrin remained.	About one-fifth of the fibrin remained.

From this set of experiments it becomes evident that alkalis destroy and do not merely suspend the active power of the ferment. From the slowness with which the digestion began, and the rate at which it proceeded, it seems that neutral salts in the solution have a deterrent effect upon it. Some previous experiments which I had made upon the behaviour of pepsin showed that it was similarly interfered with.

The experiments made upon the tube F were somewhat differently arranged. From the behaviour of the D set, the filtering seemed to make no difference to the result, so that it was omitted, but the control tubes were varied a little.

F contained the ferment in a ·5 per cent. solution of Na_2CO_3. It had been exposed to the action of the alkali for more than two days. As in the case of D, it was carefully neutralised and made up with HCl to a strength of ·2 per cent. of the acid. A measure of fibrin as before was put into it, and it was labelled F_1.

The controls were prepared as under :—

To 50 c.c. of Na_2CO_3 ·5 per cent., 5 c.c. of the dialysed ferment extract were added, and the whole neutralised at once.

Another 50 c.c. of Na_2CO_3 ·5 per cent. were neutralised, and then 5 c.c. of the same ferment extract were added. Both were made up to ·2 per cent. HCl, and a measure of fibrin placed in each. These were labelled respectively F_2 and F_3. All three were then placed on the water bath.

F_1 then contained the ferment extract that had been acted on for a time by the alkali, and some NaCl resulting from the neutralisation of the alkali.

F_2 contained the ferment that had been exposed to momentary contact only with alkali, and that at the ordinary temperature of the room; and the same amount of salt as F_1, similarly caused.

F_3 contained ferment that had not been exposed to even momentary contact with the alkali, and the same amount of salt as the others.

The subjoined Table gives the result of the action.

Time of experiment.	F_1	F_2	F_3
hours 24	No change apparent . . .	Digestion begun; more advanced than in D set of tubes after same interval, as shown by greater turbidity of liquid	As F_2.
48	A little change in the fibrin. Liquid turbid slightly. Fibrin measured seven-eighths of original bulk	Digestion advanced. About one-fifth of the fibrin only remaining	About as F_2.

The comparison of this set with the D set confirms the view that the action of the ferment is retarded by salt, for, though slower than the usual rate of action, digestion was more rapid than in the D set. The action of the alkali on the ferment in the F set was distinctly deleterious, but the destruction was not complete as in the D set.

The outcome of these experiments gives then the following answer to the question stated :—

1st. The ferment resembles pepsin in being injured by action of alkalis upon it, the amount of injury depending upon the amount of alkali present.

2nd. The action is impeded by the presence of a small quantity of NaCl in the solution, the amount of hindrance being proportional to the amount of neutral salt present. The ferment is not destroyed by NaCl as it is by Na_2CO_3.

Further experiments showed that so long an exposure to the action of the alkali was not necessary for the destruction of the ferment power. In one case an exposure of three minutes rendered it inert.

4. *What is the condition in which the ferment exists in the resting seeds;* i.e., *is it there as a zymogen or as a ferment?*

The inability of v. Gorup-Besanez to show any ferment action in the Lupin extract, contrasting so strongly with the experiments described above, raised the question of the condition of the ferment in the resting seed as compared with the germinating one. In his paper already alluded to he does not mention the condition of his seeds in this respect, but the point suggested appears to throw some light on the reason of the maintenance of the long period of quiescence. If the ferment is present in the zymogen condition during this period, there seems no difficulty in understanding why germination is deferred, for it would not proceed until set up by a development of the active ferment from the zymogen.

The method adopted in examining this point was a modification of that recently described by Langley and Edkins* in their work on the condition of the ferment in

* 'Journ. of Physiol.,' vol. 7, pp. 371–415.

the cells of the gastric glands. It depends essentially on the destruction of the zymogen and of the ferment by alkalis, and by passing a stream of CO_2 through the extract of the gland. They find that, while both these reagents ultimately destroy both pepsinogen and pepsin, the destruction of the zymogen is much more rapid than that of the ferment by CO_2, while the opposite conditions are found to obtain in the case of the alkali.

A quantity of the resting seeds was ground and freed from husks, and a watery extract made. To 20 c.c. of this, 2 c.c. of HCl of 1 per cent. strength were added, and the mixture warmed for an hour. This treatment would presumably convert any zymogen present into ferment, as it has that effect upon all the animal zymogens.

It was then carefully neutralised with ·2 per cent. Na_2CO_3, the quantity of the latter used being noted. For reference, this mixture may be called F.

To another 20 c.c. of the extract a mixture of 2 c.c. of 1 per cent. HCl and the same quantity of Na_2CO_3 that was used in the first case was at once added. This may be called Z. Both now contained the same amount of extract, and the same amount and description of salts. The only difference was that one had been warmed with acid and the other had not.

Now 5 c.c. of each of the mixtures F and Z so prepared were warmed for some time with 5 c.c. of 1 per cent. Na_2CO_3. They were then again acidified, the strength eventually being ·2 per cent. HCl, and fibrin was added. They were then put to digest in dialysers as before.

Control experiments were made by adding at once to 5 c.c. of each of the two mixtures F and Z the same amount of 1 per cent. Na_2CO_3 and of acid used in the previous cases, and these were then put to digest with fibrin in dialysers side by side with the others.

The only differences now between the last two sets of tubes were that the first two tubes had been warmed with alkali before being put to digest, while the last two had had the alkali neutralised without warming.

Simultaneous experiments were carried out on the effect of CO_2 on the extract. The gas was passed through equal quantities of the mixtures F and Z, and these were subjected to experiment with fibrin in dialysers, like the others. Controls were kept in which quantities of F and Z through which no CO_2 had been passed were tested. The dialysates were in all cases ·2 per cent. HCl.

The experiments were repeated under varied conditions as to length of exposure to the action of these different reagents.

The result of the whole series was to show that the alkali had an injurious effect upon the extract always, but that the CO_2 did no harm if the latter had been treated with acid before the gas was passed through it.

Comparing these results with those of Langley and Edkins, the following conclusions seem probable :—

1. Zymogen and not ferment exists in the resting seeds.

2. This zymogen is readily converted into ferment by the action of dilute acids.

3. The zymogen is destroyed by the action of CO_2, but the ferment is not.

4. Both zymogen and ferment are injured by the action of alkalis. There was not such a difference in the rate of injury as, from LANGLEY and EDKINS's results on pepsinogen, I expected to find.

The conditions of germination, and the preliminary changes which take place at its onset and really start it, may be deduced from these considerations. The reaction of the resting seed is neutral, and the contents of the cells are dry. On the admission of water to the cells, which invariably precedes germination, the reaction rapidly becomes acid, probably by certain decompositions made to take place in the protoplasm, whereby vegetable acids are formed. This seems not an unreasonable hypothesis, as the rapid formation of such acids in turgid cells is the only way in which the continual turgescence can be maintained, the latter depending on the osmotic equivalent of the acid. This acid, reacting on the zymogen present in the cells, developes from it the ferment, under the action of which the transformation of the resting products can readily take place.

5. *Action of the ferment on the proteids of the seed.*

The existence of the ferment and certain of the features of its action thus having been ascertained, it remained to investigate the result of its working on the proteid bodies existing in the seed. According to VINES,* these consist in the Lupin of a mixture of hemialbumose and a vegetable globulin. From the recent researches of KÜHNE and CHITTENDEN,† the body hitherto called hemialbumose by them appears to be a mixture of, in many cases, four distinct albumoses. I had not time, unfortunately, to submit VINES's hemialbumose to very elaborate investigation, but I was able to see that it is composed of at least two albumoses, which correspond fairly well with KÜHNE's protalbumose and heteroalbumose.

A watery extract of the ground resting seeds was boiled to separate the globulin which had dissolved by the assistance of the inorganic salts in the seed; it was then filtered and submitted to dialysis. None of the albumose passed the dialyser even after more than a week's exposure; the dialysate gave no biuret reaction, and a faint opalescence only with excess of alcohol. A trace of sugar passed through, but not more than the merest trace. After twenty-four hours' exposure I found that the removal of the salts had caused a copious precipitate to fall, while the solution, freed from this precipitate, gave a further precipitate on addition of HNO_3. A little of the boiled original extract, containing, as noted, a small quantity of inorganic salts, gave a fairly good precipitate on large dilution with water. All these precipitates behaved like albumoses in that they were dissolved on heating the solution in which they were suspended, and came down again as the liquid cooled.

* *Op. cit.*

† *Op. cit.*

A 10 per cent. NaCl extract of the seeds was found to give similar reactions, but to contain more of the body precipitable by dialysis or dilution of its solution. There was also extracted by this fluid a greater quantity of the globulin, as shown by the greater amount of coagulum produced by boiling.

As already mentioned, very careful and long-continued dialysis showed that neither of the proteids of the seed was capable of dialysis. The dialysate of the boiled watery extract, on concentration and slow evaporation on a glass slide, showed only here and there a needle-shaped crystal. The seed hence contains, before germination begins, two albumoses (protalbumose and heteroalbumose) and a globulin, but no peptone, nor any crystalline derivative of proteids.

Before discussing the behaviour of the ferment when mixed with these bodies for purposes of experiment, it may be well to mention certain changes which took place in a watery extract of the germinating seeds, prepared at the same time as the glycerine one used in the experiments already detailed. This watery extract when first made was turbid, and would not filter clear. It contained albumoses, as evinced by a precipitate given on addition of HNO_3 or acetic acid. After standing some days at the temperature of the laboratory, the turbidity had partially disappeared, and addition of acid failed to produce a precipitate. The ferment extracted by the water from the germinating seed had evidently performed a process of digestion of the proteids extracted coincidently with itself. A quantity of it was then submitted to dialysis for some days, and the dialysate gave a slight biuret reaction, much masked by a colouring matter that diffused out; when concentrated and evaporated on a slide, it slowly deposited crystals resembling those of asparagin.

The composition of the stored proteids having been ascertained, a quantity of the mixed albumoses was prepared from the salt extract of the resting seeds. This was chosen, as it seemed to contain the two albumoses more in the proportion in which they existed in the seed than did the watery one. The heteroalbumose is insoluble in water, and so only so much of it dissolved in the extract as was enabled to do so by the small amount of salts in the seed. This salt extract was boiled to separate the globulin, which coagulated and was filtered off. The liquid was then completely precipitated by acetic acid, and the precipitate separated by decantation and subsequent filtration, washed, and dried. It was then dissolved again and precipitated by alcohol. After standing under this for some time, it was dried and used for the purposes of the experiment, being mixed with a quantity of the ferment in new tested dialysers. The solution was made acid to an amount equal to ·2 per cent. HCl, and outside the dialysers acid of the same strength was used. The relative quantities of the two albumoses used in different experiments varied considerably, but neither was absolutely isolated in any. In all the experiments the course of the digestion was the same, and a mixture of both albumoses with the globulin behaved in the same way.

The first body formed during the digestion of the albumoses was an acid-albumin, exactly like the parapeptone described as occurring when the ferment was acting on

fibrin. This was precipitated on neutralisation, and was soluble in weak acids and alkalis. No doubt part of it was due to the action of the acid present, for a control tube, with acid only and no ferment, showed the formation of some, though not so much. The appearance of this body always struck me as peculiar, as, according to KÜHNE'S theory of proteolysis, hemialbumose is not an antecedent of such a body, but is converted at once into peptone. To this point I shall return later, merely noting here that the occurrence of the acid-albumin made me particular to see that in later experiments there was no proteid subjected to the action of the ferment but the albumoses, and then, as before, I always found this neutralisation precipitate present.

Shortly after this body had appeared the existence of true peptone was recognisable in the dialysate by the biuret reaction. I never found the dialysates give this unless ferment was present in the digesting mixture. The HCl alone had the power of producing the acid-albumin, but could take the digestion no further, or at most could produce only the merest trace of peptone.

In many cases there was mixed with the solution of the albumoses a small amount of a peculiar colouring matter, which, on the addition of caustic soda, gave a strongly marked yellowish-brown tinge to the liquid. The biuret reaction was consequently not very easily noted. I therefore adopted another test for peptone, which I found to be exceedingly delicate.* It consists in freeing the solution from all other proteids by boiling with freshly prepared ferric acetate and then adding to it acetic acid and a drop of metatungstic acid. Any peptone, which may be present, is thereby precipitated in a finely granular form. By this reaction I was able to find traces of peptone which were not discoverable by the biuret reaction, for the reason already stated. I tested the controls always by this method, at the same time as the dialysates of the proteids with ferment present, and found, as indicated by the biuret, where practicable to apply this, that a large quantity of peptone was formed by the ferment, and the merest trace only by the HCl.

With much more trouble I succeeded in showing that the ferment produced crystalline bodies, showing crystals similar to those already alluded to as being formed during the digestion of fibrin. In some cases these were mixed with other crystals, resembling those of asparagin, and in yet other cases the latter were by far the most numerous. These were deposited, on concentration of the dialysates and evaporation on glass slides. There was a difficulty in satisfying myself for some time that the crystals really came from the proteids in consequence of digestion, as my ferment extracts, being glycerine extracts of the germinating seeds, might have contained some of these bodies, although they had been themselves dialysed. Several experiments were therefore conducted to settle the point. In one series of these the dialysates were changed several times and the quantity of crystals obtainable from each observed. The fifth dialysate contained them in greater quantity than the first, which indicated a formation of them as the digestion proceeded, for if they were in the

* MARTIN: *Op. cit.*

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extract, to start with, they must have gradually diminished in each change. The most satisfactory experiment was one that was conducted on rather a large scale. A quantity of the solution of the mixed albumoses, prepared and purified as before described, was taken and acidified up to ·2 per cent. HCl, and 25 c.c. of the glycerine ferment extract were added to it. The quantity in all was 300 c.c. This was digested for a week in a large beaker on a water-bath kept constantly at about 40° C. After the expiration of this time 100 c.c. of the fluid were taken from the beaker and boiled. On neutralising it, as before, a copious precipitate fell, which was filtered off. This contained the acid-albumin formed and part of the undigested albumoses. A further quantity of these came down on addition of ammonia, and these in turn were filtered off. The clear liquid was treated then according to v. Gorup-Besanez's directions to separate the crystalline amides, as has already been described in the case of the fibrin digestion. The leucin, &c., was precipitated by basic acetate of lead, the lead compound, after washing, decomposed by SH_2, and the filtrate from this concentrated to very small bulk, when it deposited the crystals in abundance. From the precautions taken, and the amount of them obtained, these could have had no other origin than the digestion of the albumoses.

Besides these experiments made on the albumoses alone, some were carried out on whole extract, containing the globulin in addition. The course of the digestion, the disappearance of the original proteids, and the several products formed were the same in all cases.

The action of the ferment then upon the proteids in the seed was marked by the successive formation of acid-albumin or parapeptone, peptone, and leucin and asparagin in different proportions.

6. *Comparison of these results with the changes ascertainable by examination of the seed and seedling during germination.*

Following out these experiments, which were all performed apart from the seed itself, it seemed advisable to ascertain how far these successive steps could be recognised in the germinating seeds, and hence to deduce, if possible, the form in which the nitrogenous matter travels in the seedling. In his work already quoted Vines calls attention to the difference between the composition of the cell contents in the radicles and in the cotyledons of the germinating seeds, showing that when the germination took place in the dark, and the young plant was in consequence etiolated, there was much albumose and little asparagin in the cotyledons, while in the radicles asparagin alone was found. V. Gorup-Besanez also has stated that in absence of light he found leucin and asparagin in the shoots of the Vetch.

The composition of the resting seed has already been described. When germination had just begun, and the radicle was just protruding, the cotyledons were found to contain a considerable quantity of acid-albumin, but not much peptone. Seeds rather

further advanced contained more of the latter, and asparagin was also present, though in small amount. The radicles showed the absence of peptone. With metatungstic acid and acetic acid there was hardly any opalescence. When the extract of the ground-up radicles was concentrated and left in an evaporating dish, large rhombic crystals of asparagin separated out in considerable quantity. These results confirm, then, those arrived at by acting on the proteids in the laboratory. The changes in germination following the development of the ferment appear to be—

1. The formation successively of acid-albumin or parapeptone, peptone, and crystalline amides.

2. The travelling of the latter bodies to the points where plastic material is required.

It appears certain that the nitrogenous crystalline bodies are not formed at first, but that at least two intermediate bodies precede them. Also that the peptone formed is but a stage in this production, and does not leave the seat of its formation. Hence peptone is not the form in which plastic nitrogenous material travels in the seedling.

The histological changes accompanying these chemical ones are interesting. In the resting seed the proteid matter is present in the form of aleurone grains, which are small round bodies embedded in a network of protoplasm. Their outlines are sharp, and they occupy about half the whole space of the cell. In a seed in which germination has just commenced the grains are found to have become larger, probably from taking up water, and the protoplasmic network is consequently more compressed. A later stage shows the grains to have a much less distinct outline, though they retain their almost spherical shape. They are now studded with sparse granules, and appear to be dissolving from within outwards. When the radicle has attained a length of about 1·5 inch the disintegration of the grains is very marked, and the protoplasm contains empty spaces in which they formerly lay. In the actively growing seed, the radicle being 2 to 3 inches long, there is little left except the meshwork of protoplasm which, now relieved from tension, is seen to be very loose, and to contain large vacuoles.

Amylolytic Ferments.

The manner in which the stored-up cellulose is made use of in the plant has not been hitherto shown. Beyond the fact that in some seeds there is a large accumulation of it, and that then no starch appears in the reserve materials, but little is known. Judging from analogy with both starch and proteids, there seems some likelihood of the action being a ferment action.

Some seeds of the Date (*Phœnix dactylifera*) were germinated for me by Mr. Lynch at the Botanical Gardens, Cambridge. When the young plants had attained a height of about six inches they were removed, and the now partially softened seed submitted

to investigation. In his 'Text-book of Botany,' SACHS has figured the seed of *Phœnix* in the several stages of germination. From his drawings it appears that the young plant remains attached to the seed by part of the cotyledon, and by means of this it avails itself of the stored-up matter. On making a transverse section of the seed, it was seen to consist of two parts, the hard endosperm, and the soft cotyledon partly surrounded by the former. The endosperm was composed of elongated oblong cells, with enormously thickened walls, containing a very small amount of protoplasm and no starch. The cotyledon was made up of rather loosely arranged cells, containing starch granules and a very distinctive epidermis. This was composed of square cells, cuticularised, and containing a very granular deeply-staining protoplasm, but no starch.

The cotyledon was separated from the hard parts, and glycerine extracts were made of both, each being ground up as finely as possible.

The extracts were in the first instance put to digest with some ground resting seed, but after prolonged action neither of them seemed to have any effect; at least the quantity of ground cellulose had not apparently diminished.

The extract of the cotyledons was next tried on a small piece of manufactured cotton. A similar quantity of the extract was well boiled, and to it a similar piece of cotton added. After two days' digestion the former piece appeared to be attacked at the edges, where threads had frayed out. Both were now removed and boiled in FEHLING'S fluid. The one which had been in the unboiled tube became coloured with reduced copper oxide in patches, particularly in the parts which seemed softest, but the other underwent no change of colour at all. There was apparently in the extract a very small trace of a ferment which produced sugar from the cellulose, but so very little in amount as to lead to the view that the change of the cellulose in germination was not due to an isolable ferment. On looking at the cotyledon, and noticing the character of the cells covering it, which are consequently in contact with the cellulose of the endosperm, it appears more likely that the latter is gradually eaten away by this epidermis, and that the ferment only exists in these cells. This seems probable, too, from a section of the endosperm. The cells are seen to be gradually broken down where they are in contact with the cotyledon, the other cells remaining intact till they are reached by the latter.

There was no evidence that the extract of the endosperm had any action on cellulose at all. Evidently in it there is no ferment, and the change is due to agencies quite external to its cells.

Neither extract had any diastatic action.

I can confirm previous writers as to a diastatic ferment in the Lupin seeds, besides the proteolytic one.

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I may summarise my results on germinating seeds as under :—

1. There exists in the Lupin seed a proteolytic ferment, working in an acid medium, and capable of converting fibrin into peptone, leucin, and tyrosin.

2. This ferment exists in the resting seed in the form of zymogen, and the latter very readily, by the action of acids, is transformed into active ferment.

3. Its action is much interfered with by presence of too much neutral salt, and it is quite destroyed by the action of alkalies.

4. It works with extreme slowness.

5. Its action is much like that of pancreatic juice, but more hemialbumose is formed by it than by the latter.

6. It acts on the proteids in the plant in such a way as to convert them into peptone, and then into leucin, asparagin, &c.

7. The nitrogenous plastic material travels to the growing points in the form of these amide bodies, and not in that of peptone.

8. The conversion of cellulose in the date seed into plastic material is not carried out by an isolable ferment in the endosperm, but by the gradual breaking down of the latter, brought about by the epidermal cells of the cotyledon, which contain the ferment in small quantity.

The proteolytic ferment of the Lupin has considerable importance from the slowness of its action, which seems as if it would afford facility for the examination in detail of the course of proteolysis. In the foregoing paper I have called attention to two points that seem to offer a field for further investigation. The first of these is connected with the apparent formation by this ferment of considerably more "hemialbumose" from fibrin than is formed from it by either pepsin or trypsin. Whether hemialbumose be a single body, or more probably a mixture of several, it appears to approach the globulins in its reactions more nearly than does the acid-albumin or parapeptone, which is formed coincidently or later. Thus, like them, it is more freely soluble in dilute salt solutions than it is in water, in which fluid globulins are altogether insoluble. From its solution it is precipitated on saturation with the same neutral salt; at least part of it is precipitated by dialysis or dilution, just as most globulins are. In its behaviour to heat it begins to show a difference. Both globulins and hemialbumose form opalescent solutions in the cold; on raising to a temperature above 70° C., the globulins are coagulated; the hemialbumose goes into solution completely, being again thrown down on cooling. This difference is carried a step further with parapeptone, for when in solution it is quite unaffected by heating or cooling. There is, however, a difference between the latter two bodies as to the liquid in which they are soluble. In the peptones the solubility at all temperatures is still seen, and the necessity for acid or alkaline reaction in the fluid to secure such solubility has disappeared. The point suggested is, therefore, whether hemialbumose is really the first product formed, and whether it gives rise later to the other products seen, viz., parapeptone and peptone. If so, the greater quantity of it found with the

Lupin ferment may be caused by the slowness of action of the latter allowing it to accumulate before the process can be carried further.

The next point I wish to call attention to is the formation of acid-albumin or parapeptone in the digestive process brought about by the Lupin ferment in a solution of albumoses. Whether these are identical with KÜHNE's hemialbumose or not, they give reactions showing a very close connexion with it, as pointed out by VINES.* The acid-albumin formed closely resembles MEISSNER's parapeptone, and is hardly distinguishable from KÜHNE's "antialbumose." According to the theory of proteolysis advanced by the latter observer, the first decomposition of such a proteid as albumin or fibrin is the splitting of it into two groups of bodies, which he calls an "anti-" and a "hemi-" group, to each of which belongs an albumose and a peptone, and these two groups seem in their further decomposition to be independent of each other. Neither should, therefore, give rise to a member of the other group. Now parapeptone, closely resembling antialbumose, is a member of the "anti-" group, hemialbumose a member of the "hemi-" group. Consequently, if KÜHNE's theory be true, hemialbumose, when present alone, should give rise at once to peptone, and antialbumose or parapeptone should not appear.

The experiments I have narrated do not of course settle the point, for, to be quite sure, it is essential that no member of the "anti-" group should be present. Though the albumoses I worked with were carefully prepared, it seems necessary that more should be learnt about their nature and reactions, and the best way of isolating them absolutely pure, before any dogmatic statement should be made. The experiments suggest an enquiry into the fate of such perfectly isolated albumoses when acted on by trypsin, pepsin, and the Lupin ferment. Such an enquiry should embrace the animal albumoses as prepared from fibrin, and the vegetable ones that can be obtained from the seeds of plants.

Should such occurrence of parapeptone from pure albumoses be found, the point would have an important bearing on the question I raised just now as to the relative places of hemialbumose and parapeptone in the course of proteolysis.

To these questions I hope to return at a subsequent period.

* *Op. cit.*

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